Chapter 6

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1. SUMMARY

Anaerobic enrichment cultures catalysing the reductive dechlorination of chlorinated benzoic acids were obtained from three fresh-water sediments collected from seven different locations. Sub-cultures from these enrichments specifically removed *ortho*-substituted chlorine from 2,3,6-, 2,3,5- and 2,4,6-trichlorobenzoic acid, yielding chloride and 2,5-, 3,5-, and 2,4-dichlorobenzoic acids, respectively. These reductive dehalogenations were stimulated by the addition of benzoate and/or volatile organic acids. In one of these enrichments dehalogenation of *ortho*- and/or *para*-chlorine substituents was also observed from 2,3-, 2,4-, 2,5-, and 3,4-dichlorobenzoic acid, yielding 3- and 4-chlorobenzoate. Removal of *meta*-chlorines was not observed in any of the enrichments.

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2. INTRODUCTION

Many halogenated organic compounds can be degraded in anaerobic environments. Reductive dehalogenation often represents the first step under such conditions. In order to predict the fate of these chemicals in the environment and to develop techniques for the bio-remediation of polluted areas or industrial wastes, the biology and biochemistry of the microorganisms catalysing this type of reaction have been studied intensively, particularly during the last decade. As a result, reductive dehalogenation of a large number of aliphatic and aromatic compounds under a variety of environmental conditions has been described [1–3]. A general observation from these studies is that reductive dechlorination reactions performed by microorganisms appear to follow some straightforward chemical rules with respect to conversion of the chlorinated substrate [3–5]: (i) highly chlorinated compounds are more readily dehalogenated than lower-chlorinated analogues because the free-energy change of reductive dehalogenation reactions increases with an increasing degree of halogen substitution. This

principle has been demonstrated for reductive dehalogenation of chlorinated ethenes, benzenes and benzoates [6,7]; (ii) low redox-environments enhance reductive dechlorination reactions. Indeed, comparison of the ease with which reductive dehalogenation occurs under conditions with different degrees of reduction suggests a decrease in parallel with the following order of electron-acceptor utilization: carbon dioxide, sulphate, nitrate and oxygen, typically associated with increasing redox-potentials [7–14]; (iii) for dechlorination of aromatic compounds there exists a preference for certain specific positions of the chlorines substituted at the aromatic ring depending on the type and position of other substituents. For example halogen-, hydroxy- and amino-substituents, make the *ortho* and *para* positions relatively susceptible to substitution reactions. The opposite is true for carboxyl groups which are *meta*-directing groups. Thus, in anaerobic enrichments the *ortho*-chlorines (and *para*-chlorines) were removed easier, from chlorophenols and chloroanilines than those substituted at the *meta*-position. On the other hand, with chlorinated benzoic acids the order of dehalogenation seems to be *meta* > *ortho* > *para* [1,10–12,15–17].

In spite of these apparent specific preferences, examples exist of reductive dehalogenations with distinctly different specificities. Anaerobic enrichment cultures obtained from lake sediments specifically removed chlorine from the *meta*-position of halogenated benzoates [18] in agreement with the above general rules. However, subsequent studies showed that the organism responsible for this activity, *Desulfomonile tiedjei* DCB-1, is capable of removing chlorines only from the *meta*-position not only from benzoates, but also from '*ortho*- and *para*-directing' benzamides and phenols [19,20]. Moreover, enrichment cultures catalysing specific removal of *ortho*- and *para*-chlorines from some chlorobenzoates have also been described, showing the existence of bacteria with different dehalogenating capacities [11,21].

Recently, we briefly reported on the dechlorination of 2,3,6-trichlorobenzoic acid (2,3,6-TBA), a herbicide persistent in the environment [22]. The present paper reports on the properties of anaerobic enrichments obtained from various fresh-water sediments, capable of specific removal of *ortho*-chlorines from this and other chlorinated benzoic acids.

3. MATERIALS AND METHODS

3.1. Collection of inocula

Cores (10–20-cm long; 2.5-cm inner diameter) were taken from seven different fresh-water sediments, sealed with a stopper and taken to the laboratory. The cores were opened in an anaerobic glove-box and sub-samples were suspended in anaerobic medium (approx. 20% w/v) containing a mixture of various chlorobenzoates (see below). All locations, in The Netherlands, from which the sediments were collected (Table 1), had been exposed (to various degrees) to the effluent of industrial plants. River Rhine sediment was collected directly down-stream of the city of Arnhem. The Biesbosch is a fresh-water marsh located a few kilometres south of the city of Dordrecht. The two sites from which Biesbosch samples were taken were both exposed to the industrially polluted river Merwede. Four additional samples were taken from sediments of the Eems-, Winschoterdiep- and Van Starkenborgh-channel, and a small ditch, all located near the city of Groningen.

3.2. Media and growth conditions

A low-chloride minimal medium (LMM) used was described previously [22] and essentially consists of mineral salts, trace elements, vitamins, resazurin and a phosphate buffer (pH 7.0). For primary enrichments the medium was supplemented with yeast extract and peptone (0.1 g/l) and volatile organic acids (1.2 mM acetate, 0.4 mM propionate, 0.2 mM butyrate, 0.05 mM 2-methylbutyrate, 0.05 mM isobutyrate, 0.05 mM valerate and 0.05 mM isovalerate). Benzoate and a mixture of different chlorobenzoates (Table 1) were added to final concentrations of 0.3 to 0.5 mM each. Medium components were sterilized by autoclaving or filtration (vitamins and chlorobenzoates). Media were prepared in screw-capped or butyl-stoppered bottles under a N_2-atmosphere

and reduced with Na_2S (approx. 0.5 mM). Incubations were done statically at 30°C in the dark.

3.3. Analytical methods

Chlorinated benzoic acids were analyzed through capillary gas chromatography (GC) using the operation conditions as described previously [22]. Preparation of samples was slightly modified. To 0.5 ml of a sample 1 mM of cinnamate was added as an internal standard. After addition of 1.5 ml 0.25 N H_2SO_4, chlorobenzoates were extracted in 0.5 ml $CHCl_3$ for analysis. Samples for capillary gas chromatography-mass spectrometry (GC-MS) were methylated and extracted in ethyl-acetate. GC-MS analysis was done as described by Parsons et al. [23]. Quantification of chlorobenzoates was by comparison with commercially obtained standards. Methane concentrations in head-space samples were also determined with a gas chromatograph [24] and chloride was measured colorimetrically with NaCl as the standard [25].

3.4. Chemicals

All chemicals used were of analytical grade except for 4-amino-3,5-dichlorobenzoic acid (95% pure) and 2,3,6-trichlorobenzoic acid (72% pure) which were shown to contain (by GC) some additional di- and tri-chlorobenzoic acids and unidentified compounds [22].

4. RESULTS

4.1. Enrichment of reductive dechlorinating activity of chlorinated benzoic acids in sediment slurries

In November 1989 fresh-water sediments were collected from six different locations in The Netherlands. Using strictly anaerobic techniques, slurries were prepared in a minimal medium (LMM) containing a volatile organic acids mixture, some yeast extract (0.01% w/v), benzoate (0.5 mM), a mixture of nine chlorinated benzoic acids (0.3–0.5 mM of each) and wet sediment (approx. 20% w/v).

In all sediment slurries benzoate was degraded within 2 months of incubation (Table 1). Benzoate degradation coincided with the production of significant amounts of methane. In cultures inoculated with sediment obtained from the river Rhine and from the Biesbosch the concentration of 2,3,6-trichlorobenzoate (2,3,6-TBA) started to

Table 1

Capacities of fresh-water sediments obtained from various locations to degrade chlorobenzoates anaerobically [a]

Source of inoculum [b]	Chlorobenzoate isomers added											
	Ba	2-	3-	4-	2,4-	2,5-	2,6-	3,5-	2,3,6-	4A3,5-	2,3-	3,4-
River Rhine (Arnhem)	+	–	–	–	–	–	–	–	+	–	n.t.	n.t.
Biesbosch Site 1 (Dordrecht)	+	–	–	–	–	–	–	–	+	+	n.t.	n.t.
Eems-channel (Groningen)	+	–	–	–	–	–	–	–	–	–	n.t.	n.t.
Van Starkenborgh-channel (Groningen)	+	–	–	–	–	–	–	–	–	–	n.t.	n.t.
Winschoterdiep (Groningen)	+	–	–	–	–	–	–	–	–	–	n.t.	n.t.
Small ditch (Groningen)	+	–	–	–	–	–	–	–	–	–	n.t.	n.t.
Biesbosch Site 2 (Dordrecht)	+	–	–	–	+	+	–	–	+	–	+	+

[a] Ba = benzoate; 2-, 3-, 4- = 2-, 3-, 4-chlorobenzoate; 2,4-, 2,5-, 2,6-, 3,5-, 2,3-, 3,4- = 2,4-, 2,5-, 2,6-, 3,5-, 2,3-, 3,4-dichlorobenzoate; 4A3,5- = 4-amino-3,5-dichlorobenzoate; 2,3,6- = 2,3,6-trichlorobenzoate; + = more than 50% removal within 2 years of incubation; – = less than 20% removal; n.t. = not tested.

[b] All samples were collected in November 1989, except 'Biesbosch Site 2' which was collected in December 1990.

decline within 1–2 months of incubation, reaching a concentration < 10 μM in a period of 6 months. Without exception the disappearance of 2,3,6-TBA was parallelled by the appearance of a product which was identified as 2,5-dichlorobenzoate (2,5-DBA) by means of capillary gas chromatography-mass spectrometry.

No significant disappearance of (chloro)benzoates was observed within 3 years of anaerobic incubation in control incubations (sterile media) and in enrichments, inoculated with samples from sites other than Rhine and Biesbosch.

The rate of reductive dechlorination in river Rhine and Biesbosch slurries increased after repeated depletion and re-addition of 2,3,6-TBA and benzoate, indicating the enrichment of dechlorinating bacteria (Table 2). In the absence of benzoate dechlorination of 2,3,6-TBA to 2,5-DBA remained incomplete but could be resumed by benzoate addition.

In the Rhine sediment enrichment 2,3,6-TBA was the only chlorobenzoate to be metabolized. However, in the slurry inoculated with Biesbosch sediment the concentration of 4-amino-3,5-dichlorobenzoate had also dropped by more than 90% after 2 months of incubation. No identifiable primary product, e.g. 4-amino-3-chlorobenzoate or 3,5-dichlorobenzoate, was detected. This activity was not maintained after repeated additions of 4-amino-3,5-dichlorobenzoate (together with benzoate).

Table 2

Enrichment of 2,3,6-trichlorobenzoate degrading capacity in Rhine and Biesbosch sediment slurries after repeated additions of 2,3,6-TBA and benzoate

Addition	Initial rate of 2,3,6-TBA dechlorination (μM/day) [a]	
	Rhine	Biesbosch
1	3.2	0.5
2	2.2	2.2
3	7.6	8.7
4	7.6	16.7

[a] Measured as the rate of formation of 2,5-DBA.

In December 1990 a second sediment sample was obtained from the Biesbosch at a location (Site 2) approx. 2 km from the location sampled in November 1989 (Table 1). For this enrichment the medium was slightly modified. It not only contained 5 mM benzoate instead of 0.5 mM, but 2,3- and 3,4-dichlorobenzoate were added to the mixture of nine chlorobenzoates used previously. Again, within several months the concentration of 2,3,6-TBA declined in parallel with an increase in the concentration of 2,5-DBA (Table 1). In addition to 2,3,6-TBA the concentrations of 2,3-,

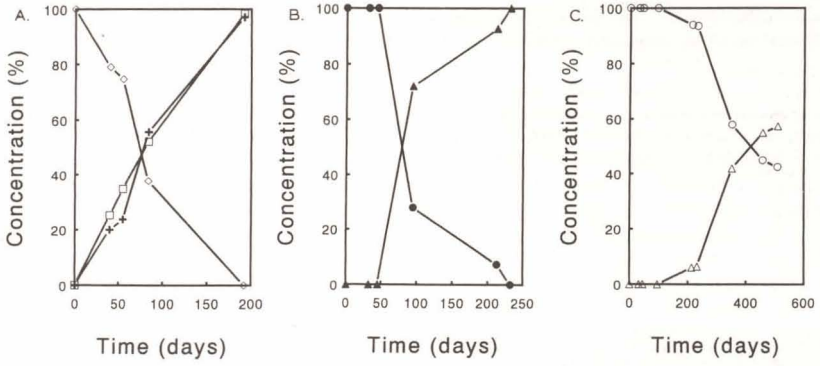

Fig. 1. Reductive dechlorination of (**A**) 2,3,6-trichlorobenzoate (◇) to 2,5-dichlorobenzoate (+) and chloride (□) and of (**B**) 2,3,5-trichlorobenzoate (●) to 3,5-dichlorobenzoate (▲), and of (**C**) 2,4,6-trichlorobenzoate (○) to 2,4-dichlorobenzoate (△) in anaerobic batch cultures obtained from Biesbosch sediment. Chlorinated substrates were added in concentrations of 0.8–1.2 mM.

2,4- and 3,4-dichlorobenzoate dropped to < 10 μM within a period of 16 months. When all the 2,3,6-TBA was metabolized, the 2,5-DBA started to disappear. Together with the decrease of these dichlorobenzoates, the concentration of 3-chlorobenzoate increased stoichiometrically. Also some 4-chlorobenzoate was produced. The concentration of 2-chlorobenzoate did not change significantly.

In none of the enrichments obtained in the present study did the sum of the concentrations of all chlorobenzoates decrease during incubation (> 2 years) to a value lower than added to the slurry at the start of the experiment. Apparently, dechlorination of mono-chlorobenzoates to benzoate and subsequent mineralization did not take place.

4.2. Some properties of 2,3,6-trichlorobenzoic acid dechlorinating bacteria in transfers from primary enrichments

After 7 months of enrichment, samples of sediment slurries 'Rhine' and 'Biesbosch Site 1' were inoculated (1–4% v/v) in media containing 2,3,6-TBA, benzoate and volatile organic acids. Reductive dechlorination by sub-cultures from Rhine sediment was lost after subsequent transfers. In contrast, cultures derived from Biesbosch sediment maintained dechlorinating activity for at least three transfers in the laboratory. In all cultures, disappearance of 2,3,6-TBA coincided with the formation of stoichiometric amounts of 2,5-DBA and chloride (Fig. 1A). No production of 2,6-DBA was detected, demonstrating that the reductive dechlorination of 2,3,6-TBA was very specific ($> 99\%$) at the *ortho*-position.

Table 3

Effect of medium composition on relative rate of reductive dechlorination of 2,3,6-trichlorobenzoate in the primary transfers from the Biesbosch-enrichment into fresh media

Yeast extract + peptone (g/l)	Benzoate (1.2 mM)	Volatile organic acids [b]	t_{50} [a] (days)
0.1	+	+	98
1	+	+	76
1	–	+	73
1	–	–	309

[a] t_{50}: time needed (days) for 50% dechlorination of 2,3,6-TBA to 2,5-DBA. Initial 2,3,6-TBA concentration was 0.9–1.2 mM.

[b] See MATERIALS AND METHODS for composition of organic acids mixture.

The influence of varying concentrations of different medium additions on dechlorination is shown in Table 3. In media containing only 0.1 g/l of yeast extract and peptone the addition of benzoate and/or volatile organic acids was needed for complete dechlorination of 2,3,6-TBA to 2,5-DBA. No significant reductive dechlorination was observed when the concentration of 2,3,6-TBA was raised to 10 mM, or in media which contained $H_2 + CO_2$, formate, acetate, lactate or pyruvate as potential carbon and electron donors, but in which no benzoate or the organic acid mixture were present (data not shown).

If actively 2,3,6-TBA dechlorinating cultures were inoculated in media containing yeast extract and peptone (1 g/l), benzoate (1 mM) and the volatile organic acids mixture no dechlorination was observed with other chlorobenzoates within an incubation period of > 15 months. The following mono- and dichlorobenzoates were tested: 2-, 3-, 2,3- 2,5-, 2,6- or 4-amino-3,5-(di)chlorobenzoate. However, two other trichlorobenzoates, i.e. 2,3,5- and 2,4,6-trichlorobenzoate, were dechlorinated. Production of 3,5-dichlorobenzoate from 2,3,5-TBA (Fig. 1B) and of 2,4-dichlorobenzoate from 2,4,6-TBA (Fig. 1C) was observed with a t_{50} of approx. 80 and 420 days, respectively. Formation of additional (mono- or di-) chlorobenzoates was not observed ($< 1\%$), substantiating that reductive dechlorination of these trichlorobenzoates was also very specific for *ortho*-chloride substituents.

5. DISCUSSION

Reductive dehalogenation of chlorinated benzoates has been reported many times in the recent literature. Although removal of chloride from all positions of the aromatic ring has been observed these studies seem to indicate that *meta*-substituted chlorobenzoates are more susceptible to biodegradation under anaerobic conditions

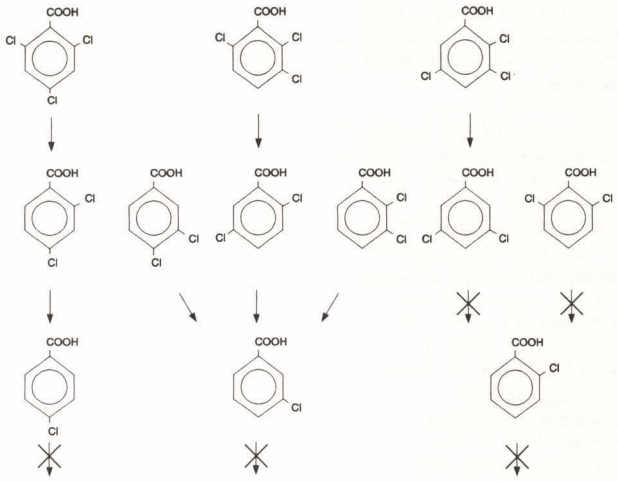

Fig. 2. Likely sequence of chlorobenzoate dechlorination observed in various enrichments obtained from fresh-water sediments.

than *ortho*- or *para*-substituted chlorobenzoates [2,10,11,18]. However, the present study has demonstrated the enrichment of microbial activity specific for removal of chlorine from the *ortho*-position with no activity for *meta*-substituted chlorines.

The capacity of enrichments obtained from various fresh-water sediments to metabolize chlorobenzoates anaerobically differed considerably. All enrichments readily utilized benzoate and produced methane, but actively dechlorinating enrichments were only obtained from three out of seven samples: only those which were collected from river Rhine and Biesbosch sediments, known to be polluted with chlorinated aromatic compounds [26]. One can speculate that dechlorinating bacteria have been selected for in such environments, especially if such organisms benefit from this type of reactions. Indeed, generation of energy was actually demonstrated for the dechlorinating bacterium *Desulfomonile tiedjei* DCB-1 which is able to couple the reductive dechlorination of 3-chlorobenzoate with the generation of a proton-motive force and subsequent ATP-synthesis [27,28].

Notwithstanding the fact that dechlorinating enrichments could be maintained in laboratory cultures, further detailed experiments with these cultures were hampered by the very low rate of 2,3,6-trichlorobenzoate (2,3,6-TBA) degradation. Some stimulation of the dechlorination occurred after addition of volatile organic acids and/or benzoate suggesting that these compounds could act as a source of reducing equivalents for reductive dechlorination of 2,3,6-TBA. Volatile organic acids, benzoate and fermentation products (e.g. H_2) formed from them have previously been shown to enhance reductive dechlorination of various halogenated aromatic and aliphatic compounds [12,29–32].

A tentative scheme of dechlorination reactions, based on the disappearance and appearance of chlorobenzoates observed in the enrichment cultures in the present study, is given in Fig. 2. The fact that disappearance of 2,3-, 2,4-, 2,5- and 3,4-dichlorobenzoates and 2,3,5-, 2,3,6- and 2,4,6-trichlorobenzoates resulted in the production of *meta*- or *para*-substituted analogues, but not in the formation of 2-chlorobenzoate, demonstrated the removal of *ortho*- (2,3-, 2,4-, 2,5-DBA

and 2,3,5-, 2,3,6-, 2,4,6-TBA) or *para*-chlorines (3,4-DBA) from these compounds. To the best of our knowledge, this is the first report indicating reductive dechlorination of 2,3-DBA and 2,3,5- and 2,4,6-TBA.

Reductive dechlorination of the *ortho*-substituted chlorine from 2,4-DBA yielding 4-chlorobenzoate has been described before by Van den Tweel et al. [33]. Interestingly, however, this was demonstrated for an aerobic bacterium. Removal of *ortho*-chlorine has also been indicated in anaerobic enrichments metabolizing 2-chlorobenzoate [21].

Dechlorination of 3,4-DBA at the *para*-position has been reported once, however, typically loss of the *meta*-chlorine from this compound occurred [1,11]. Although the removal of 4-chlorobenzoate by anaerobic (denitrifying) enrichments has been described, no evidence was presented that this was the result of reductive dehalogenation [21].

Although in the present study removal of *ortho*-chlorines from 2,5-DBA and 2,3,6-TBA was demonstrated, Suflita et al. [34] reported removal of *meta*-chlorines from these same compounds. *Desulfomonile tiedjei* DCB-1, the organism responsible for these reactions also *meta*-dechlorinated 3-, 3,5- and 4-amino-3,5- (di) chlorobenzoates [20]. Surprisingly these compounds appeared persistent in our enrichments.

It is not clear why in the present study monochlorinated benzoic acids, formed during reductive dehalogenation of di- and tri-chlorinated substrates were not further metabolized. Indeed, mono-chlorinated compounds are usually less easily dechlorinated reductively than those containing several chlorine substituents, but these reactions are nevertheless thermodynamically favourable. The observation that enrichments obtained from different locations displayed large variation with respect to the pattern of degradation of chlorobenzoates has been reported before and seems to reflect the fact that bacterial populations with diverse catabolic abilities are indigenous in these habitats [10,11]. This makes it very difficult to predict in detail the fate of these compounds in natural environments.

Microbial communities with diverse specificities of removal of chlorine from the *ortho*-, *meta*- or *para*-position of chlorinated phenols or biphenyls have also been obtained from various sources [16,35,36]. Combining such populations could result in more complete dechlorination. For mineralization of poly-chlorinated compounds, substituted at multiple positions, it is thus important to acquire bacteria with supplemental dechlorinating capacities.

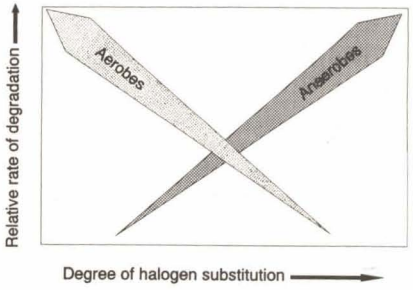

Fig. 3. Trends in the degradation of halogenated compounds. Poly-halogenated compounds are best degraded by anaerobic bacteria, whereas compounds containing only few halogens are most readily mineralized by aerobes.

The observed pattern of sequential dehalogenation of poly-halogenated compounds, resulting in accumulation of less halogenated products was not only found for the anaerobic degradation of chlorinated benzoates [7]. This phenomenon was also observed during degradation of chlorinated ethenes, benzenes, phenols, benzoates, biphenyls and anilines and appears to be a characteristic of the anaerobic mineralization of halogenated compounds [1,3,12,15,17,34,37–41]. Fortunately, such compounds with few halogen substituents are usually more readily degraded by aerobic bacteria [3,42]. Figure 3 represents the general pattern which emerges from the currently available data on aerobic and anaerobic biodegradation of halogenated aliphatic and aromatic compounds. Recent reports describe the successful application of combining the activity of anaerobic and aerobic bacteria for the degradation of poly-halogenated compounds [22,43]. Although these experiments were done under labo-

ratory conditions they are encouraging for the development of systems allowing the biological removal of these xenobiotics from polluted environments.

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